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Over-expression of *NPR1* gene in *Brassica juncea* Leads to Resistance Against *Alternaria brassicae* Infection

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ABSTRACT: *Alternaria* blight is one of the serious diseases of *Brassica juncea* causing 45-58% loss in the yield. In the present days the most common method to control this biotic issue is the chemical method. However, use of these chemicals causes huge damage to the human health, plant health and eventually polluting our ecosystem. Thus, manipulating defense regulatory genes [e.g. NoPR1 (*NPR1)* gene] is one of the safest strategies which is being followed internationally to manage this disease. Previously in order to develop resistance against *Alternaria brassicae*, nineteen putative transgenic lines of *B.juncea* having *NPR1* gene under control of 35S promoter were developed. In the present study, these transgenic lines were confirmed for gene integration, expression and its effect on resistance against *Alternaria*. Gene integration was confirmed by PCR with *NPTII* primers. In order to reconfirm the *NPR1*gene integration, PCR was done using 35S forward primer and *NPR1* reverse primer. All the nineteen putative lines were found to be positive. Further, RT-PCR with *NPR1* primers was done to check levels of gene expression. Eleven lines namely (1, 5, 6, 7, 8, 9, 11, 12, 14, 16, 18) were found to be over expressing *NPR1* by about 5 fold over non transgenic control while other eight lines (2, 3, 4, 10, 13, 15, 17, 19) did not show such high expression (only 2 fold). Seven lines encompassing both high *NPR1* expressing and not so high *NPR1* expressing, were analyzed for disease resistance. Resistance was scored in terms of time of onset of symptoms, lesions number and lesion size. The symptoms of infection were observed on day 3 after inoculation in control plants whereas those in transgenic plants symptoms were observed on day 7 after inoculation. The levels of resistance *in-vitro* varied from 2.0 to 2.5 fold as compared to the control. *In-vivo* assays revealed 2 to 3 fold resistance in transgenic plants as compared to control plants. It was found that expression level of *NPR1* is directly related to levels of resistance.

Keywords: *NPR1*, Transgenic, Fungus, Resistance, *Brassica*, *Alternaria*.

INTRODUCTION

Brassica juncea is economically very important crop because of its high oil content and superior oil quality. Its oil content typically varies between 36 and 42 %, of this; average oil recovery is approximately 35 % (Srinivasan 2005). The major factors for poor yield in *Brassica juncea* are insects and diseases. The crop is susceptible to a number of pathogenic diseases among which the most important and devastating fungal disease is *Alternaria* blight, caused by *Alternaria brassicae.* This causes 57% loss in the yield (Directorate of Economic Survey 2022). Plant breeders have been trying albeit without success so far to develop disease resistant line for many years through conventional plant breeding methods. Unfortunately, there is no source of resistance against this pathogen among the sexually compatible relatives of *Brassica juncea*. The absence of resistance genes within crossable germplasm of *Brassica* necessitates use of genetic engineering strategies to develop genetic resistance against this pathogen.

Therefore, efforts are being made done in order to have sufficient knowledge about the genes induced during infection and their regulation measures. This is particularly important for *Brassica juncea* as there is very little information available on defense mechanisms in this crop. All over the world, biotechnology-based fungal disease resistance programs are being carried out for developing resistance against *Alternaria brassicae*. Besides using the strategies of over-expressing genes for antifungal compounds, and using R gene, manipulation of regulatory genes which encodes signal proteins required for downstream antifungal genes is also an important strategy for disease resistance (Cao *et al*., 1997). One such regulatory gene is *NPR1*gene.Expression of *NPR1* is induced by pathogen infection or treatment with defense-inducing compounds such as salicylic acid (SA) etc. In the absence of pathogen infection or SA signal, *NPR1* protein is present in an oligomeric form through

intermolecular disulfide bonds sequestered in the cytoplasm and is excluded from the nucleus. After pathogen recognition, increased SA levels induce a bi-phasic change in the cellular redox environment (Mou *et al.,* 2003). Following an initial increase in the reduction potential, plant cells attain a more reducing environment because of the accumulation of antioxidants. Under these conditions, *NPR1* is reduced to a monomeric form that accumulates in the nucleus, bind TGA-type transcription factors and then ultimately activate the expression of PR protein genes. Additional proteins such as *NIMIN1* interact with *NPR1* in complexes that are mediated by specific protein-protein interaction domains within the *NPR1* protein sequence (Ekengren *et al.,* 2003; Despres *et al.,* 2003; Mou *et al.,* 2003; Thurow *et al.,* 2005; Weigel *et al.,* 2005; Xu *et al.,* 2006). The transgenic plants developed with *NPR1* have also been reported in other economically important crop species, such as tomato, rice, banana, sugar-beet and cotton (Liu *et al.,* 2002; Lin *et al.,* 2004; Chern *et al.,* 2005; Kuykendall Kuykendal *et al.,* 2007). In the present study molecular and phenotypic level were analyzed of transgenics having *NPR1* under control of 35S promoter which was developed earlier in the laboratory (Ali *et al*., 2017).

MATERIALS AND METHODS

Biological material and growth conditions. Nineteen transgenic *Brassica juncea* lines which were developed in the Plant Pathogen Interacting laboratory of National Institute for Plant Biotechnology were used in this study.For negative control untransformed *Brassica juncea* plants were used. Plants were grown in pots containing compost mixture in a growth chamber (temperature at 18°C night and 25°C day time, photoperiod under 14 h light and 10 dark, relative humidity at 60-70%) of the National Phytotron Facility, IARI, New Delhi (Fig. 1). *Alternaria* culture was grown on Radish Root Extract Sucrose Medium (RRESM) and Potato Dextrose Agar (PDA) media at 22°C under 70-80% Relative humidity for 14 hrs photoperiod.

Fig. 1. T_0 Transgenic plants of *Brassica juncea* having *NPR1* gene.

DNA isolation and PCR amplification. Leaf samples were collected from 1 month old plants from date of sowing. Total genomic DNA was extracted from young leaf tissue of putative transgenic plantlets and untransformed wild type *Brassica juncea*plants by CTAB method (Murray and Thompson 1980, Fig. 2).

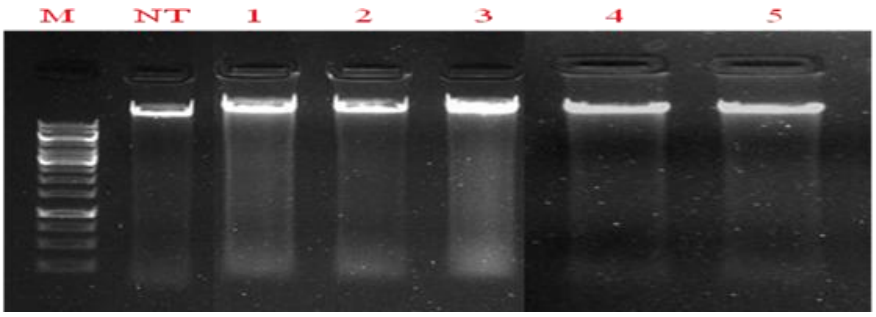

Fig. 2. DNA isolated from transgenic and non-transgenic plants. Lane1:-M (Marker-1kb DNA Ladder). Lane2:- Non Transgenic plants. Lane3-7:-Transgenic Plants.

Two different PCR reactions were carried out, one by using (NOS promoter) forward and NPTII reverse primers from *NPTII* gene (henceforth will be called *NPTII* primer)and another using 35S forward and *NPR1* Reverse primers. The primers used for amplification are as follow-

Primer: *NPTII* (BangaloreGenei)

Forward primer: 5'-AGGCGATAGAAGGCGATGCGC-3'

Reverse primer: 5'-CAATCGGCTGCTCTGATGCCG-3'

CaMV 35S Forward: 5' –GGAAAAGGAAGGTGGCTCCTAC-3'

NPR1 Reverse 5'-TGTCCCGGGTAACTCTGTAACAC- 3'

The PCR products were then electrophoresed and analyzed.

Plant RNA isolation and RT-PCR. RNA isolation was carried out by Trizol method (Sigma-Aldrich, USA). Using young leaf tissues of putative transgenic and untransformed wild type *Brassica juncea*var. varuna plantlets were used for RNA isolation (Fig. 3).

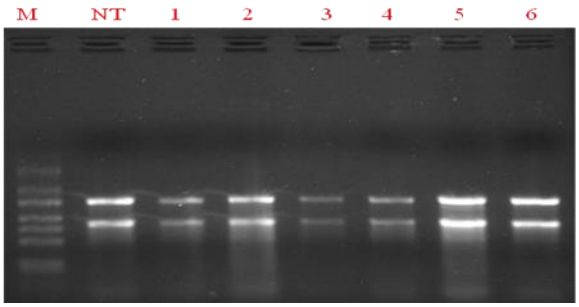

Fig. 3. RNA isolated from transgenic and non-transgenic plants. Lane1:- M (Marker-1kb DNA Ladder). Lane2:- Non Transgenic plants. Lane3-8:-Transgenic Plants.

Super Script TM III One-Step RT-PCR System with Platinum® *Taq*DNA polymerase, provided by Invitrogen, was carried out using gene-specific *NPR1* primers to analyze the expression of the transgene in the putative *Brassica juncea* transgenic plantlets. RNA isolated from untransformed *Brassica juncea* and RT-PCR reaction mixture without any RNA (but with water) were used as negative controls. The primer pairs used were:-

NPR1 Gene

Forward: - 5' -TACTGACCTCCTGAAACGTGAG- 3'

Reverse: - 5' -TGTCCCGGGTAACTCTGTAACAC- 3'

Phenotypic analysis of transgenic plants infected *in-vitro by* spore suspension. Petriplates having moist filter paper (two layers) were used for *in-vitro* studies. Healthy *Brassica juncea* leaves were plucked out and were kept in the petriplates infected with the spore suspension (1.5×10^6 spores/ml). 10µl of spore suspension was placed onto five to six randomly selected places on the leaf surface and this was done on 5-6 different leaves of same plant. The leaves were incubated in a BOD incubator for 10 hour dark and 14 hour light, moisture content was maintained by periodically watering the filter paper. Incubation was done till the symptoms were observed and evaluation was done after 7 days from inoculation.

Phenotypic analysis of transgenic plants infected *in-vivo by* spore suspension. *Brassica juncea* plants were grown in Phytotron chamber under conditions mentioned above Section. Healthy leaves were infected with the spore suspension (1.5×10^6 spores/ml). 10µl of spore suspension culture was placed onto five to six randomly selected places on the leaf surface and this was done on 5-6 different leaves of same plant. Plants were watered once a day till the symptoms appeared. Evaluation was done after 7 days from inoculation.

RESULTS

Molecular analysis of putative transgenic lines of *Brassica juncea.* The young leaf tissue of the putative *Brassica juncea* transformants at the rooting stage were subjected to molecular analysis by PCR and RT-PCR to analyze the integration and expression of the transgenic, respectively. We observed the result and found the resistance development in all the transgenics against *Alternaria brassicae.*

Genomic DNA isolation and polymerase chain reaction. Genomic DNA was extracted using CTAB method from the leaf tissue of the plantlets and was checked on 1% agarose gel (Fig. 2). To check integration of *NPR1* gene, NOS promoter forward and *NPTII* reverse primers were used. Nevertheless to check the integration of *NPR1* gene, *NPR 1*primer could not be used as amplification of endogenous gene as it would interfere with the results. To avoid that forward primer of 35S promoter and reverse primer of *NPR1* gene were used. Genomic DNA extracted from non transgenic *Brassica juncea* was used as a negative control. PCR products obtained with *NPTII* primers showed a band of 1 Kb (Fig.3&4). PCR product obtained with 35S forward and *NPR1* gene reverse primer showed a 900 bp band (Fig. 5&6).As expected, both the bands were missing in the non-transgenic plants. All 19 plants analyzed were found to be PCR positive for both *NPTII* and *NPR1*primers.

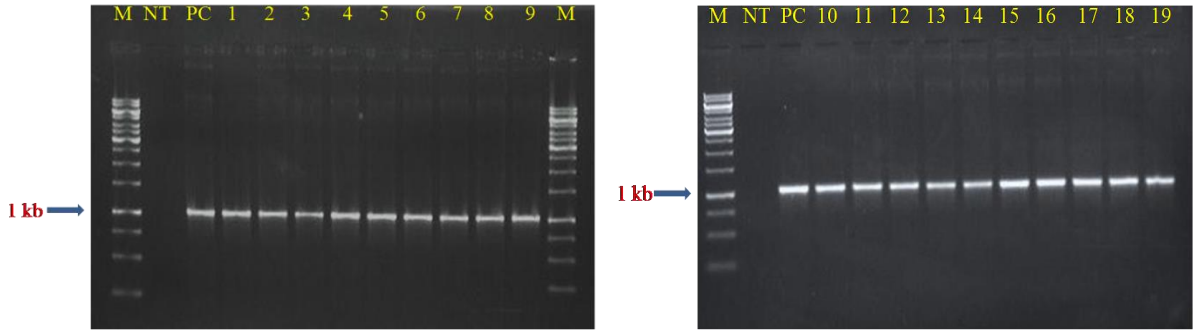

Fig. 3-4: Analysis of transgenic plants for gene integration by PCR with NOS promoter forward and *NPTII* Reverse Primers. Lane1:- M (Marker-1 kb DNA Ladder). Lane2:-NT (Non Transgenic plants). Lane3:- PC (Positive control) pBI-121(Binary vector) having *NPR1*gene. Lane4-12 & 4-13:-Transgenic Plants.

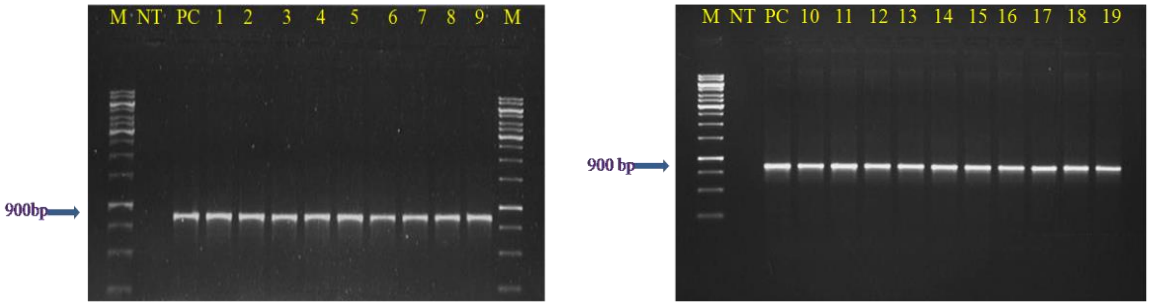

Fig. 5-6. Analysis of transgenic plants for gene integration by PCR with 35S promoter as the forward primer and *NPR1* as the reverse primer. Lane1:- M (Marker-1 kb DNA Ladder). Lane2:- NT (Non Transgenic plants). Lane3:- PC (Positive control) pBI-121(Binary vector) having *NPR1* gene. Lane4-12 &4-13:-Transgenic Plants.

Total RNA isolation and RT-PCR. Total RNA was isolated by using Triazol method from all transgenic lines as well as nontransgenic plant of *Brassica juncea*.RNA was checked on 1% agarose gel prepared in 1X MOPS solution (Fig.3). Then RNA was converted into c-DNA by using fermentas reverse transcriptase enzyme. Finally RT-PCR was carried out to check the expression level of *NPR1*gene using *NPR1* forward and reverse primers (Fig.7&8).It was found that in lines 1,5,6,7,8,9,11,12,14,16,18 expression level was as high as 5 fold compared to control plants while in other lines it was comparatively low but still higher than control (2 fold). Actin gene was used to see that the equal amount of RNA loaded in each well.

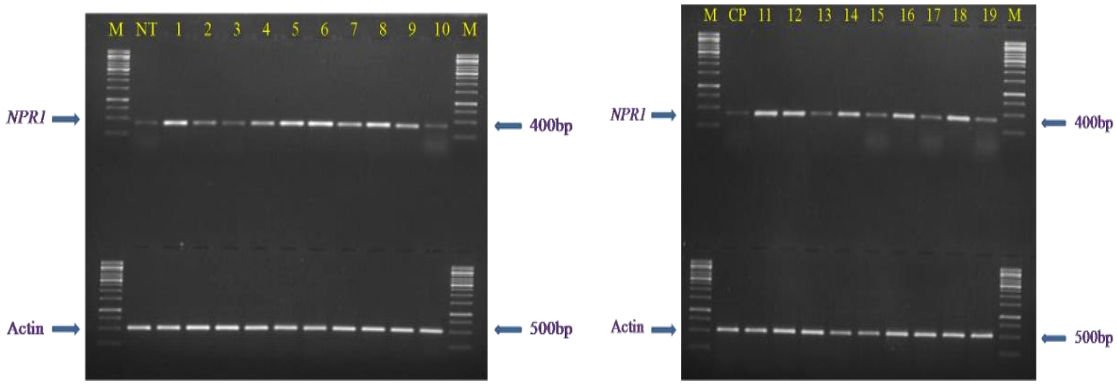

Fig. 7-8. Analysis of putative transgenic plants of *B. juncea* for gene expression by RT-PCR is using *NPR1* primer and Actin primer. Lane1:-M (Marker-1 kb DNA Ladder). Lane2:-NT (Non Transgenic plants).Lane3-12 & 3-11:- Transgenic Plants

Phenotypic analysis of *B. juncea* transgenics by *in vitro* and *in vivo* methods. Fungal spore's suspension was made and *in vitro* and *in vivo* analysis was done to analyze the resistance of plants for *Alternaria brassicae*.

***In-vitro* analysis.** Evaluation was done post 7 days of inoculation. Transgenic lines 1, 5,7,12 (showing 5 fold over-expression of *NPR1*) and lines 10, 13, 15 (showing only 2 fold) were selected for phenotypic analysis. Symptoms were observed as early as 3-4 days after inoculation in untransformed plants whereas in transgenic plants symptoms were observed after 5-6 days from inoculation. The typical symptoms of *Alternaria* infection i.e. middle necrotic area by a yellow hallows (concentric rings) were seen. It was observed that lines showing a higher level of over expression of *NPR1* also showed more resistance with respect to lesion number and lesion size (Fig. 9&10). It implies that the level of *NPR1* expression is correlated with the level of resistance.

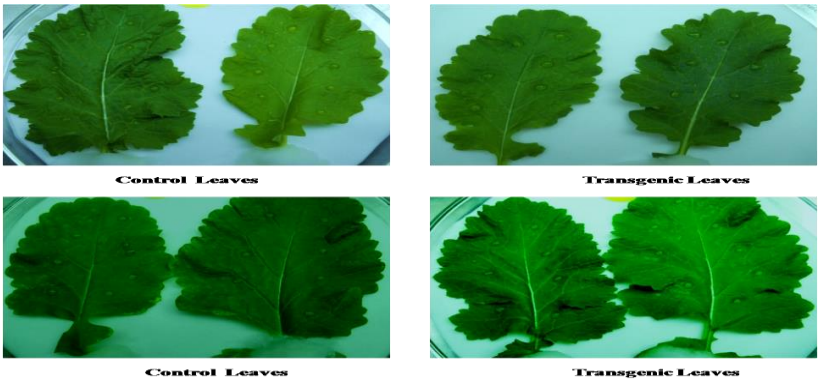

Fig. 9. Screening for resistance in *Brassica juncea* transgenic plants against *Alternaria brassicae* by *in-vitro* method. (Droplets of spore suspension are visible on leaves on day 0)

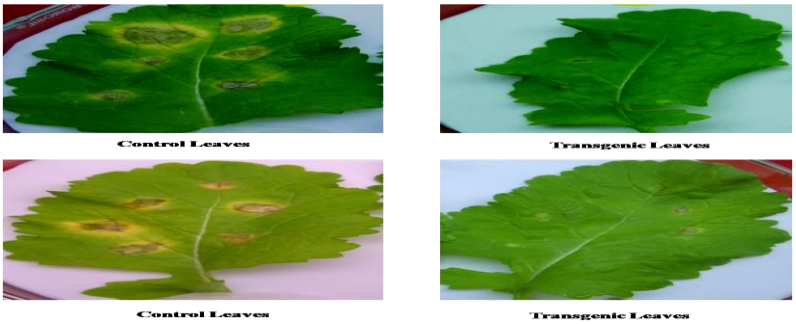

Fig. 10. Screening for resistance in *Brassica juncea* transgenic plants against *Alternaria brassicae* by *in-vitro* method. (Symptoms were more pronounced in control plants compared to that in transgenic lines after 7 days of inoculation)

As shown in the table1, *NPR1* transgenics showed a reduced number of symptoms as compared to the control plants. Different transgenic plants showed different level of resistance. Besides visual screening, resistance was scored by lesion number and lesion size. In control plants the average lesion size was 7.9 mm while in transgenic plants the lesion size varied from 2.7 to 6.6 mm. Although there appeared 6 lesions per leaf in control, only 3-4 lesions per leaf were observed in transgenic plants (Table 1).

Table 1: Phenotypic analysis of resistance in transgenic plants against *Alternaria* infection *in-vitro*.

T_0 Line	Leaf No.	Number of Lesion per Leaf	Average No. of Lesion per Leaf	Lesion Size (mm)						Average Lesion Size (mm)	Average Lesion size of Four Leaves (mm)
1	1 2 3 4	6 4 5 3	4	7 6 9 4	6 4 2 2	5 5 5 3	4 3 6 -	3 - 3 -	2 - - -	4.5 4.5 5 3	4.2
5	1 2 3 4	3 2 3 3	3	4 2 2 3	3 3 3 2	3 - 4 3	- - - -	- - - -	- - - -	3 2.5 3 2.6	2.7
7	1 2 3 4	2 6 3 2	3	4 4 6 3	5 2 4 2	- 6 2 -	- 4 - -	- 2 - -	- 3 - -	4.5 3.5 4 2.5	3.6
10	1 2 3 4	4 2 4 3	3	8 6 7 6	12 5 8 5	9 - 3 9	7 - 4 -	- - - -	- - - -	9 5.5 5.5 6.6	6.6
12	1 2 3 4	2 3 4 2	3	4 4 2 3	3 2 3 2	- 3 2 -	- - 3 -	- - - -	- - - -	3.5 3 2.5 2.5	2.8
13	1 2 3 4	3 2 6 4	4	7 5 3 5	3 7 4 4	2 6 2 8	- - 4 4	- - 3 -	- - 2 -	4 6 3 5	4.5
15	1 2 3 4	6 5 4 6	5	15 6 9 6	8 4 7 12	6 5 1 4	5 3 2 3	4 4 - 10	2 - - 5	6.6 4.4 4.7 6.6	5.5
Control (Varuna)	1 2 3 4	6 5 6 6	6	15 5 8 13	14 6 9 12	10 7 10 7	9 4 5 9	7 8 6 5	5 - 7 4	10 6 7.5 10	7.9

***In-vivo* analysis.** Evaluation was done 7 days post inoculation. For *in-vivo* analysis also, same lines were chosen as those during *in-vitro* analysis (1, 5, 7, and 12) with higher expression of *NPR1* and 10, 13, 15 with low level of *NPR1* expression. As can been seen in (Table2) that lesion size was reduced from 6.2 mm in untransformed control plants to 2 mm in *NPR1* high expressing lines (e.g. 1 and 5) and to 3 mm in comparatively low expressing lines (e.g. 13 and 15). Thus, we observed that there is direct relation between extent of *NPR1* expression and reduction in number and size of the lesions (Fig. 11& 12). From Table 2 it is seen that different transgenic lines show better resistance to *Alternaria in-vivo* studies compared to *in-vitro* studies. This can be attributed that leaves attached to the plants showed better resistance mechanisms.

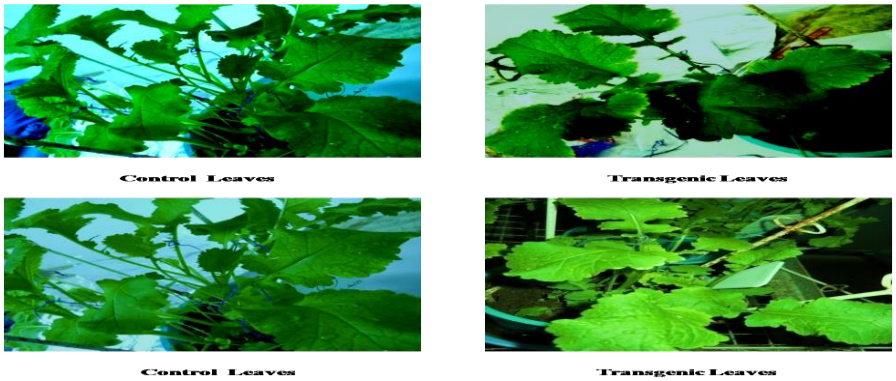

Fig. 11. Screening for resistance in *Brassica juncea* transgenic plants against *Alternaria brassicae* by *in-vivo* method. (Droplets of spore suspension are visible on leaves on day 0)

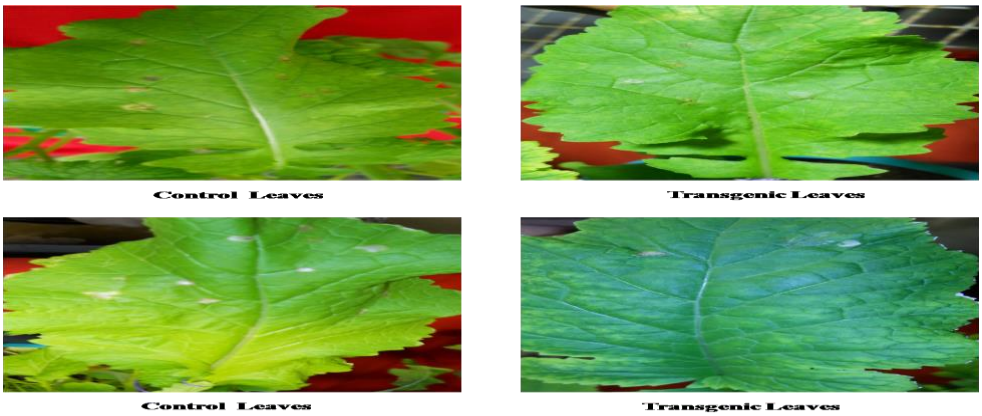

Fig. 12: Screening for resistance in *Brassica juncea* transgenic plants against *Alternaria brassicae* by *in-vivo* method (After 7 day of infection, symptoms were more pronounced in control plants compared to that in transgenic lines).

Table 2: Phenotypic analysis of resistance in transgenic plants against *Alternaria* infection *in- vivo*.

T_0 Line	Leaf No.	Number of Lesion per Leaf	Average No of Lesion per Leaf	Lesion Size (mm)						Average Lesion Size (mm)	Average Lesion size of Six Leaves (mm)
1	1	6	2	5	5	3	2	3	4	3.6	2
	2	2		3	2	-	-	-	-	2.5	
	3	0		-	-	-	-	-	-	-	
	4	4		3	2	2	3	-	-	2.5	
	5	0		-	-	-	-	-	-	-	
	6	1		3	-	-	-	-	-	3	
5	1	5	3	4	3	2	3	2	-	2.8	2
	2	5		2	4	2	2	1	-	2.2	
	3	4		3	4	3	2	-	-	3	
	4	4		5	2	2	3	-	-	2.5	
	5	0		-	-	-	-	-	-	-	
	6	1		3	-	-	-	-	-	3	
7	1	6	3	5	5	2	3	2	3	3.3	2.3
	2	2		5	2	-	-	-	-	3.5	
	3	2		3	2	-	-	-	-	2.5	
	4	5		3	2	3	2	3	-	2.6	
	5	0		-	-	-	-	-	-	-	
	6	2		2	2	-	-	-	-	2	
10	1	6	4	5	2	3	2	3	4	3.2	2.8
	2	5		3	2	1	2	3	-	3	
	3	6		5	3	6	2	3	4	3	
	4	5		3	2	-	5	4	-	4	

	5 6	1 0		4 -	- -	- -	- -	- -	- -	4 -	
12	1 2 3 4 5 6	4 2 4 1 0 5	3	5 2 3 2 - 3	3 3 5 - - 5	2 - 1 - - 2	2 - 2 - - 3	- - - - - 2	- - - - - -	3 2.5 2.8 2 - 3	2.2
13	1 2 3 4 5 6	2 4 6 5 0 3	4	5 5 3 4 - 5	3 2 2 5 - 4	- 3 2 3 - 3	- 2 5 2 - -	- - 2 2 - -	- - 3 - - -	4 3 2.8 3.2 - 4	2.8
15	1 2 3 4 5 6	4 3 0 5 0 4	4	4 4 - 3 - 4	2 2 - 2 - 3	3 5 - 3 - 5	4 - - 2 - 3	- - - 2 - -	- - - - - -	3.2 3.6 - 2.4 - 3.7	3
Control (Varuna)	1 2 3 4 5 6	4 6 5 6 5 4	5	6 7 6 7.4 5.4 7.4	5.4 8 5 7 5 5.9	7 7.2 4 6 4.4 6.9	5 7.7 5 8.4 6 5.8	- 7.2 4.9 7.7 - 6.8	- 7.8 - 6.7 - 6.7	5.8 7.4 4.9 7.2 5.2 6.6	6.2

DISCUSSION

Besides over-expressing antifungal compounds and transferring R genes for developing disease resistance, one other strategy is to over-express defense regulatory gene. Among several regulatory gene *No PR1* (*NPR1*) is of the significant regulatory gene. It induces an array of defense gene down in the signal transduction pathway (e.g. PR1, chitinase, glucanase, defensins etc.). Over-expressing *NPR1* gene amounts to over-expressing different PR genes and hence is equivalent to gene pyramiding.

Nineteen putative transgenic *Brassica juncea* lines having *NPR1* gene under the control of 35S promoter were analyzed for gene integration, expression and disease resistance. While analyzing for *NPTII* gene integration, NOS promoter forward and *NPTII* reverse primers were used. *NPR1* primers could not be used to check for *NPR1* integration as the endogenous *NPR1* gene might interfere with the results. Thus to avoid that forward primer of 35S promoter was chosen and reverse primer of *NPR1* gene was used.

To study the gene expression, *NPR1* primers were used for RT-PCR. While 11 lines were over-expressing *NPR1* gene by 5 fold as compared to control, 8 lines were showing only 2 fold over-expression. These differential expressions of *NPR1* among different transgenic lines are caused by position effect (integration of the gene at different places in different transgenic lines).Corresponding to differential expression of *NPR1*, there were different levels of resistance to *Alternaria* among different transgenic lines. For example *in-vitro* studies, lines 5 and 12 were showing more resistance (size of lesions and number of the lesion) and these lines also showed high level of *NPR1*gene induction. Line 10 and 15 were showing less resistance (although higher than control) corresponding to their low level of *NPR1*gene expression. As compared to lesions size, lesions number did not showed significance difference. This could be because; all the leaves were inoculated by spore suspension at five to six selected places. During *in-vivo* infections also line 1, 5 and 12 showed better resistances as compared to the line 10, 13 and 15. It corresponded to their *NPR1* level of expression. It was also seen that lines which show reduced lesion number also display reduced size of the lesion. So, it can be inferred that over-expression of defense regulatory gene *NPR1* leads to improved resistance in *Brassica juncea* against *Alternaria brassicae*. Most of the transgenic lines show better resistance to *Alternaria in-vivo* studies compared to *in-vitro* studies. This could be because leaves were still attached to the plants *in-vivo* assay and resistance mechanisms might be working better. Over-expressing *NPR1* is known to induce several defense genes and hence is equivalent to gene pyramiding. Therefore, transgenic plants analyzed in the present study need also be analyzed for over-expression of other antifungal genes in future.

CONCLUSIONS

Nineteen putative transgenic *Brassica juncea* lines having *NPR1* gene under the control of 35S promoter were developed in the Plant Pathogen Interacting laboratory of National Institute for Plant Biotechnology. The present study was molecular analysis of putative transgenic lines for gene integration and expression and phenotypic analysis for disease resistance against *Alternariabrassicae*infection.DNA was isolated from control and transgenic plants and PCR was done with *NPTII* primers (for kanamycin resistance) and with 35S promoter forward primer and *NPR1* reverse primers. All the nineteen lines were found to be positive. The study of gene expression was accomplished by isolating RNA from control and transgenic plants and doing RT-PCR with *NPR1* primers. Eleven lines were found to over- express *NPR1* gene by 5 fold while eight lines were found to over express *NPR1*gene by 2 fold.

Transgenic plants were evaluated after inoculation of one month old plants through *in-vitro* (by detached leaf

method) and *in-vivo* (on the whole plant) methods. Improved resistance was measured by delay in onset of symptoms and number and size of the lesions. Appearance of symptoms was delayed by 2 days in transgenic plants as compared to the control plants. Number and size of the lesions were reduced from 6 and 7.9 mm in control plants to 3 and 2.7 mm in transgenic plants respectively during *in vitro* studies. In *in-vivo* studies also number and size of the lesions were reduced from 5 and 6.2 mm in control to 2 and 2 mm in transgenic plants respectively. It was also seen that transgenic lines showing fewer lesions also displayed small size lesions. From the present study, it was concluded that integrating the *NPR1* gene and over-expressing it, leads to improved resistance in *B. juncea* against *A. brassicae.*

FUTURE SCOPE

Present study clearly shows the role of NPR1 to enhance the resistance against biotic challenge. Thus, NPR1 gene could be used to develop new resistant genotypes against *Alternaris brassicae* in *B. juncea* to mitigate the produce and economical losses.

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Conflict of Interest. None.

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